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Evaluation of the Therapeutic Usefulness of Botulinum Neurotoxin B, C1, E, and F Compared with the Long Lasting Type A

BASIS FOR DISTINCT DURATIONS OF INHIBITION OF EXOCYTOSIS IN CENTRAL NEURONS*

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Patrick G. Foran‡, Nadiem Mohammed‡, Godfrey O. Lisk‡, Sharuna Nagwaney‡, Gary W. Lawrence‡, Eric Johnson§, Leonard Smith¶, K. Roger Aoki‖, and J. Oliver Dolly‡**

From the ‡Centre for Neurobiochemistry, Department of Biological Sciences, Imperial College, London SW7 2AZ, United Kingdom, the §Department of Food Microbiology and Toxicology, Madison, Wisconsin 53706-1187, the ¶Toxinology Division, United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21702-5011, and ‖Allergan Inc., Irvine, California 92623-9534

Seven types (A–G) of botulinum neurotoxin (BoNT) target peripheral cholinergic neurons where they selectively proteolyze SNAP-25 (BoNT/A, BoNT/C1, and BoNT/E), syntaxin1 (BoNT/C1), and synaptobrevin (BoNT/B, BoNT/D, BoNT/F, and BoNT/G), SNARE proteins responsible for transmitter release, to cause neuromuscular paralysis but of different durations. BoNT/A paralysis lasts longest (4–6 months) in humans, hence its widespread clinical use for the treatment of dystonias. Molecular mechanisms underlying these distinct inhibitory patterns were deciphered in rat cerebellar neurons by quantifying the half-life of the effect of each toxin, the speed of replenishment of their substrates, and the degradation of the cleaved products, experiments not readily feasible at motor nerve endings. Correlation of target cleavage with blockade of transmitter release yielded half-lives of inhibition for BoNT/A, BoNT/C1, BoNT/B, BoNT/F, and BoNT/E (≫31, ≫25, ~10, ~2, and ~0.8 days, respectively), equivalent to the neuromuscular paralysis times found in mice, with recovery of release coinciding with reappearance of the intact SNAREs. A limiting factor for the short neuroparalytic durations of BoNT/F and BoNT/E is the replenishment of synaptobrevin or SNAP-25, whereas pulse labeling revealed that extended inhibition by BoNT/A, BoNT/B, or BoNT/C1 results from longevity of each protease. These novel findings could aid development of new toxin therapies for patients resistant to BoNT/A and effective treatments for human botulism.

Seven immunologically distinct serotypes of botulinum neurotoxin (BoNT)[1] (A–G) from *Clostridium* botulinum are homologous proteins consisting of a heavy and light chain linked by an essential disulfide and noncovalent interactions that specifically block the release of acetylcholine at the neuromuscular junction (reviewed in Refs. 1–3). BoNTs cause botulism, the majority of human outbreaks being caused by types A, B, or E (1); however, they are remarkably useful as therapeutic agents (see below). The striking potency of the toxins and their cholinergic selectivity arise from their multiple domains mediating: (i) targeting to motor nerve endings via high affinity interaction with ecto-acceptors located exclusively thereon (4, 5) and (ii) endocytosis (6) followed by translocation of a LC-containing moiety into the cytosol. Their LCs are Zn^{2+}-dependent endoproteases that selectively cleave single peptide bonds (except for BoNT/C1; see below) in one of three SNARE proteins that constitute the components of a ternary complex responsible for vesicle docking/fusion during regulated exocytosis (7). Synaptosomal-associated protein of 25 kDa (SNAP-25) (8) is proteolyzed by BoNT/A, BoNT/C1, and BoNT/E at separate sites near the C terminus: Gln^{197}–Arg^{198}, Arg^{198}–Ala^{199}, and Arg^{180}–Ile^{181}, respectively (3). Another plasmalemmal protein, syntaxin1 (STx1) (reviewed in Ref. 9), is also cleaved by BoNT/C1, and synaptobrevin, a synaptic vesicle protein (Sbr) (10, 11) is cleaved by BoNT/B, BoNT/D, BoNT/F, BoNT/G, and tetanus toxin (TeTx). BoNT/A- or BoNT/E-truncated SNAP-25 (termed $SNAP\text{-}25_A$ or $SNAP\text{-}25_E$, respectively) remains membrane-bound, but release is inhibited; in the case of $SNAP\text{-}25_A$, some assembly and disassembly of the ternary complex can still occur (12, 13). Truncation of STx1 or Sbr by the requisite BoNT results in detachment of their cytosolic domains.

When applied locally to humans for the treatment of dystonias (reviewed in Ref. 14), BoNT/A, BoNT/B, and BoNT/E cause neuromuscular paralysis for more than 4 months, ~2 months, or <4 weeks, respectively (15, 16); the limited results available for type C1 suggest a duration less than or equal to that of BoNT/A (17). It is unclear why the recovery times in rodents are shorter and yet show the same rank order (1–2 months (BoNT/A), 21 days (BoNT/B), 7 days (BoNT/F), and 4 days (BoNT/E)) (18, 19).[2] Insight has been gained into the sequence of events involved in the protracted resumption of neurotransmission in BoNT/A-poisoned motor endplates by monitoring synaptic function in individually identified nerve endings of living mice (18). This showed that the transient appearance of functional nerve sprouts mediates a partial return of neuromuscular function, with full recovery relying on the originally affected endings reacquiring the ability to mediate chemical transmission. In chromaffin cells, the persistence of BoNT/A

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** To whom correspondence should be addressed. Tel.: 44-208-393-4438; Fax: 44-208-224-7629; E-mail: dr.oliver@dolly.fsworld.co.uk.

[1] The abbreviations used are: BoNT, botulinum neurotoxin; DIV, days *in vitro*; KRH, Krebs-Ringer-HEPES; LC, light chain; Sbr, synaptobrevin; SNAP-25, 25-kDa synaptosomal-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; STx1, syntaxin1; TeTx, tetanus toxin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

[2] Meunier, F. A., Lisk, G., Sesardic, D., and Dolly, J. O. (2003) *Mol. Cell. Neurobiol.*, in press.

protease for many weeks contributes to the extended inhibition of secretion; also, SNAP-25_A has been shown to be inhibitory (21–23). Of particular interest, Eleopra *et al.* (16) reported that co-treating human endplates with BoNT/A and BoNT/E results in a more rapid recovery of neuromuscular function, equivalent to that of BoNT/E alone, prompting many scientists (16, 22, 24)[2] to suggest that both proteases have equivalent lifetimes in the motor nerve ending and the prolonged paralysis by BoNT/A arises from slow replacement of SNAP-25_A. Accordingly, Meunier *et al.*[2] observed that type E hastens the removal of inhibitory SNAP-25_A from BoNT/A-treated mouse neuromuscular synapses by converting it to SNAP-25_E, which is replaced rapidly; thus, resumption of synaptic transmission is accelerated.

In this study, biochemical analyses (not practical with motor nerve endings or isolated motoneurons; see "Discussion") were performed on cultured cerebellar neurons to quantify the half-lives of toxin inhibition and the rates of turnover of SNAREs and their toxin-cleaved products. Although noncholinergic, these neurons provide a useful model for studying the intracellular fate of BoNTs, because we observed the same relative durations of neuroparalytic actions of BoNT/A, BoNT/B, BoNT/C1, BoNT/E, and BoNT/F as measured in motor nerves *in vivo* (see above). In addition, these homogeneous cerebellar neurons are very susceptible to BoNTs and could be obtained in sufficient numbers for these quantitative measurements. In this way, we have extended earlier findings (22, 25) and explained how exocytosis can be blocked for dissimilar periods by the different BoNT serotypes.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and general reagents were supplied by Sigma-Aldrich. N2 supplement and serum (extensively dialyzed before use) were from Invitrogen, [^{14}C]glutamine was from Amersham Biosciences, and [^{35}S]methionine was from ICN. Monoclonal antibodies selectively reactive with STx1, *Sbr*2, or SNAP-25 were purchased from Sigma-Aldrich (clone HPC-1), Synaptic Systems (clone 69.1), and Sternberger Monoclonals Inc. (clone SMI-81), respectively. Igs specific for SNAP-25 (C-terminal residues 195–206), *Sbr*1 and 2 (a 62-mer peptide residues 32–94 of human *Sbr*2; termed HV62), synaptotagmin1 (its last 20 amino acids), and SNAP-23 (11 residues at the C terminus) were produced in rabbits and affinity-purified as before (26, 27). Anti-glial fibrillary acidic protein Igs were a gift from Dr. G. P. Wilkin. All of the neurotoxins used were of >95% purity, fully nicked as assessed by SDS-PAGE and protein staining, and exhibited maximal lethalities in mice. Pure BoNT/F M complex (BoTx/F) was supplied by WAKO Chemicals (Osaka, Japan). All work with BoNTs was performed using approved, strictly enforced safety precautions.

Preparation and Maintenance of Cerebellar Granule Neurons: Exposure to BoNTs and Assay of Glutamate Release—These cells were dissociated from the cerebella of 7–8-day-old rats (28) and suspended at ~1 × 10^6/ml in 3 parts of basal Eagle's medium and 1 part of 40 mM HEPES-NaOH, pH 7.3, 78.4 mM KCl, 37.6 mM D-glucose, 2.8 mM $CaCl_2$, 1.6 mM $MgSO_4$, and 1.0 mM NaH_2PO_4, as well as 1x N2 supplement, 1 mM L-glutamine, 60 units/ml penicillin, 60 μg/ml streptomycin, and 2% (v/v) horse dialyzed serum. An aliquot (1 ml) of this cell suspension was added to each of 16-mm-diameter poly-D-lysine coated well (*i.e.* 24 -format) and cytosine-β-D-arabinofuranoside (40 μM) added after culturing for 20–24 h in 5% (v/v) CO_2; the neurons were maintained by replacement every 10 days with the same freshly prepared medium. This preparation is reported to contain largely (90–95%) glutamatergic interneurons (29), up to 6% γ-aminobutyric acid-ergeric cells and 3% glial fibrillary acidic protein-containing astrocytes (30), controlled using an anti-mitotic agent. Where specified, the neurons were exposed to toxin (0.2-μm filter sterilized) in culture medium for 24 h; unbound toxin was removed by three washes (over 10 min) with Krebs-Ringer-HEPES (KRH; 20 mM HEPES-NaOH, pH 7.4, 128 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM D-glucose, and 0.05 mM mg/ml bovine serum albumin, pH 7.4), and then the culture medium was replaced.

For measurement of transmitter release, the neurons were washed four times with O_2-pregassed KRH and incubated with 0.4 ml of the latter buffer containing 0.25 μCi/ml [^{14}C]glutamine (*i.e.* a glutamate precursor) (31); all of the steps were performed at 37 °C. After 45 min, the neurons were washed thrice briefly and twice for 5 min each time prior to a 5-min incubation in KRH containing 1.4 mM Ca^{2+} or 0.5 mM EGTA (*i.e.* to assess Ca^{2+}-dependent, resting release); the aliquots were retained for measurement of [^{14}C]glutamate content by ion exchange HPLC analysis and scintillation counting (31). A modified KRH buffer containing 50 mM KCl (with a reduced NaCl content of 83 mM to maintain osmolarity) and 1.4 mM Ca^{2+} or 0.5 mM EGTA was added for a 5-min stimulation period. The amounts of Ca^{2+}-dependent [^{14}C]glutamate released into basal and K^+-stimulated samples were measured as above, expressed as percentages of the total cell content, and the evoked component was calculated. Glia from rat cerebella were cultured in medium lacking an anti-mitotic agent and containing 10% (v/v) fetal calf serum (nondialyzed); 2 days before use, 0.1 mM glutamate and glycine were added to kill any residual neurons (32).

The use of extensive washing steps removes sick or dead cells that detach from wells, precluding a significant contribution to the experimental measurements. The healthy state of the cells remaining bound is indicated by several important criteria, including their abilities to efficiently perform multiple energy-dependent steps and attain up to 90% proteolysis of SNAREs by very low BoNT concentrations.

Pulse Labeling and SNARE Immunoprecipitation—Neurons ~ 5 × 10^6/well (35-mm diameter) were washed four times with O_2-pregassed KRH and incubated in a modified culture medium retaining all of the above-noted additives except lacking serum and L-methionine but instead containing [^{35}S]methionine (50–100 μCi/ml). After 4 h, the neurons were washed twice and harvested immediately or "chased" in conventional medium supplemented with 0.25 mM unlabeled L-methionine. Washed neurons were detergent solubilized for 30 min (0.6 ml) using 2% (w/v) CHAPS and 2% (w/v) *n*-octyl β-D-glucopyranoside in 20 mM HEPES-NaOH, pH 7.5, containing 10 mM EDTA, 150 mM NaCl, 1% (w/v) bovine serum albumin, and 2% (v/v) of a protease inhibitor mixture (P8340; Sigma). All of the steps were performed at 0–4 °C. Insoluble material was removed by centrifugation at 15,000 × *g* for 40 min, and the extracts were incubated for 3–4 h in an end-over agitator with the relevant anti-SNARE Ig-protein A-agarose complex (10 μg of Ig/50 μl of resin). Resin was collected by centrifugation (5 s at 100 × *g*) and washed eight times over 30 min (1 ml each) with solubilization buffer lacking the protease mixture and containing only 0.1% (v/v) each of CHAPS and *n*-octyl β-D-glucopyranoside. Nonreducing SDS-PAGE sample buffer was added to the agarose slurry and heated at 80 °C for 20 min. The radioactive immunoprecipitated SNAREs were subjected to SDS-PAGE, fixed, treated with Amplify™ (Amersham Biosciences), dried for fluorography, and detected using Hyperfilm MP™. Control experiments found that BoNT/A, BoNT/B, BoNT/E, or BoNT/F pretreatments had no effect on protein synthesis, by measuring the amounts of radioactivity incorporated into precipitatable protein relative to toxin-free controls (measured by scintillation counting; data not shown).

Immunoblotting and Quantitation of Antigens—Immediately after assaying transmitter release, the cells were solubilized in 1% (w/v) SDS in 20 mM HEPES-NaOH, pH 8.5, containing 20 mM EDTA plus 150 mM NaCl; the total protein was quantitatively isolated using chloroform-methanol precipitation (outlined in Ref. 27). For optimal resolution of intact SNAP-25 from its toxin-truncated products, the samples were subjected to SDS-PAGE using NOVEX ™ 12% Bis-Tris gels and a MOPS-based buffer system (Invitrogen). The proteins were electrotransferred and immunoblotted, as detailed previously (27), with detection by anti-species-specific Igs conjugated to horseradish peroxidase and visualization by enhanced chemiluminescence. The blots were densitometrically scanned, and the bands were quantified using image analysis software (Scion Image for Windows); the standard curves of the amounts of SNARE plotted against band intensity were constructed to allow accurate quantitation.

RESULTS

Recovery of Neuroexocytosis from BoNT/E- or BoNT/F-inhibited Rat Cerebellar Neurons Is Rapid and Coincident with the Respective Reappearance of Intact SNAP-25 or Sbr2—Initially, the central neurons were shown to be suitable for studying the dynamics of SNAREs and neuroexocytosis. Cerebellar granule cells, maintained under partial depolarization (31, 33), developed over time into mature neurons, establishing numerous neurite contacts (Fig. 1*A*) that are known to form functional synapses (34, 35). Immunoblotting revealed a minimal content of glial fibrillary acidic protein-reactive astrocytes but an abundance of STx1, *Sbr*2, SNAP-25, and synaptotagmin 1, being

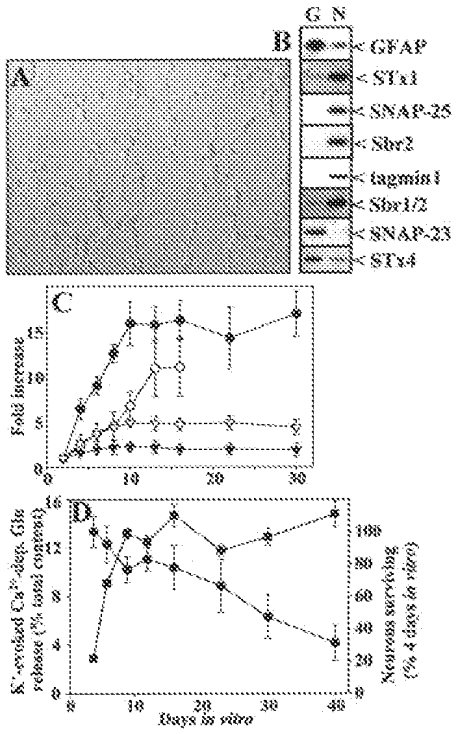

FIG. 1. **Elevation of Ca^{2+}-dependent transmitter release and concomitant increase in SNARE contents during development of cerebellar neurons: lack of SNAP-23.** *A*, the rat neurons (prepared as detailed under "Experimental Procedures") were cultured for 14 DIV before visualization by interference contrast microscopy. *B* and *C*, equal amounts (10 μg) of protein from neurons (*N*) and glia (*G*) (see "Experimental Procedures") were subjected to immunoblotting with the antibodies specified; primary Igs were detected using horseradish peroxidase-labeled secondary Igs and visualized by enhanced chemiluminescence. Sbr isoform 2 was specifically detected with a monoclonal 69.1 while both isoforms (*Sbr*1/2) were visualized with anti-HV62 (see "Experimental Procedures"). The temporal expression of STx1 (▼), SNAP-25 (▽), *Sbr*2 (●), or synaptotagmin1 (○) was quantified by densitometric scanning of blots, and the fold increase in the immunoreactivity of each was expressed relative to the signals present at 2 DIV (*C*, only). *D*, The cells were loaded with ^{14}C-labeled glutamine for the quantitation of Ca^{2+}-dependent evoked release of glutamate (■ as outlined in "Experimental Procedures") and assessment of neuron survival by microscopy (●). The data are the means ± S.D. (n = 3 or 4).

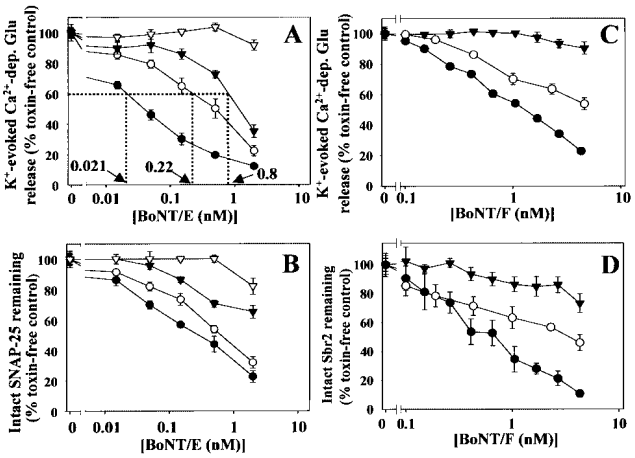

FIG. 2. **BoNT/E and BoNT/F cause short-lived blockade of transmitter release that coincides with the reappearance of intact SNAREs: determination of the half-lives of inhibition.** *A*, after incubation of cerebellar neurons (7 DIV) for 24 h at 37 °C in culture medium containing BoNT/E (*A* and *B*) or BoNT/F (*C* and *D*), the washed cells were assayed immediately at day 0 (●), or the medium was replaced and the culture was maintained for 2 (○), 4 (▼), or 7 (▽ days in the absence of toxin after BoNT/E treatment or 3 (○) or 7 (▼) days following BoNT/F. *A* and *C*, evoked transmitter release was quantified (means ± S.D.; n = 3 or 4). *B* and *D*, equal amounts of protein were immunoblotted using SMI-81 Ig (SNAP-25) and anti-HV62 Ig (*Sbr*2) to assess the extents of SNARE proteolysis (means ± S.D.; n = 3), calculated after densitometric scanning of the blots. The extrapolated toxin concentrations causing equivalent blockade of transmitter release at various recovery times were used to calculate the $t_{½}$ of inhibition (example shown in *A*).

much more prominent in neurons than glia (Fig. 1*B*). In contrast, SNAP-23, a BoNT/A-insensitive but BoNT/E-cleavable non-neuronal SNAP-25 homologue (27, 36, 37) was apparently absent from the granule cells but present in glia (Fig. 1*B*). Interestingly, the expression of STx1 and, particularly, SNAP-25, *Sbr*2, and synaptotagmin1 increased markedly during neuron development and synaptogenesis (Fig. 1*C*) concomitant with the maturation of the evoked exocytotic response, reaching a plateau at 10–13 days in culture (Fig. 1*D*). Importantly, for the purpose of studying SNARE function and the persistence of BoNT action, sufficient quantities of the developed neurons remained viable for several weeks (Fig. 1*D*), allowing the dynamics of SNARE expression and turnover to be investigated (see below).

Dose dependences for inhibition of evoked exocytosis were measured in granule cells exposed to BoNT/E or BoNT/F for 24 h; a maximum inhibition of ~80% and ~90% was seen, with corresponding losses of intact SNAP-25 (BoNT/E; Fig. 2, *A* and *B*) or *Sbr*2 (BoNT/F; Fig. 2, *C* and *D*). The different concentrations of the two toxins necessary to give 50% blockade of evoked release, 43 pM and 1.35 nM respectively, concur with their disparate specific neurotoxicities in mice (data not shown). After BoNT/E removal by washing, the extent of the initial inhibition (*i.e.* day 0) and the amounts of $SNAP\text{-}25_E$ decayed progressively over 2–7 days (Fig. 2, *A* and *B*); the observed restoration to the preintoxication level of intact SNAP-25 indicated loss of the protease activity. Similarly, type F-treated neurons regained the majority of their *Sbr*2, coincident with a fairly fast return to the initial level of exocytosis (Fig. 2, *C* and *D*). In both cases, exocytosis appeared to resume more rapidly from a partial blockade, with minimal inhibition of exocytosis and only traces of SNAP-25 or *Sbr*2 cleavage being noted at 7 days (Fig. 2, *B* and *D*). Replenishment of intact SNAREs coincided with resumption of exocytosis. From the dose dependence, a half-life of the duration of inhibition ($t_{½\ INH}$) by each toxin was determined by monitoring reduction in the extent of blockade of exocytosis at different times after initial exposure to a given concentration of toxin. In the case of BoNT/E, the concentrations required to yield a 40% inhibition of exocytosis at 0, 2, and 4 days after the removal of toxin were 0.021, 0.22, and 0.80 nM, respectively (Fig. 2*A*). When these values were subjected to first order decay analysis, a $t_{½\ INH}$ of 0.70 ± 0.15 days (mean ± S.D.; n = 6) was calculated; a second series of experiments yielded a comparable value. The mean of both experiments was 0.73 ± 0.11 days (mean ± S.D.; n = 12; Table I). Similarly, analyses of data from two recovery experiments with BoNT/F yielded a mean $t_{½\ INH}$ of 1.76 ± 0.28 days (Table I). It is assumed that these $t_{½\ INH}$ values represent a combination of the times required for cellular removal of the toxin protease activity and synthesis of functional intact SNAREs.

BoNT/A-induced Blockade of Transmitter Release from Cerebellar Neurons Lasts Much Longer than That Caused by Type B—Neurons treated for 24 h with BoNT/A yielded dose-dependent inhibition of transmitter release up to a maximum of 65% blockade at 25 pM (Fig. 3*A*), accompanied by proteolysis of up to 90% of the SNAP-25 (Fig. 3*B*). More extensive inhibition of the Ca^{2+}-dependent release could not be achieved, even with 2 nM toxin (data not shown; see "Discussion"); it is notable that only 10 pM yielded ~ 50% blockade of the inhibitable component (Table I). After toxin was washed away, no significant recovery

TABLE I
Potencies and durations of inhibition of exocytosis by BoNTs in cerebellar granule neurons

Purified nicked toxin	Concentration causing 50% blockade of transmitter release[a]	$t_{1/2}$ of inhibition[b]
	pM	*days ± S.D.*
BoNT/A	10	≫31[c]
BoNT/B	100	9.84 ± 2.12
BoNT/C1	13	≫25[c]
BoNT/E	43	0.73 ± 0.11
BoTx/F	1350	1.76 ± 0.28
TeTx	6.5	Not studied

[a] These values were determined from detailed concentration dependency studies plotted in Figs. 2–4.
[b] The $t_{1/2}$ is calculated by subjecting the time-dependent decreases of inhibition to first order decay analysis.
[c] No significant diminution in the extent of inhibition was noted at the listed times, in several experiments.

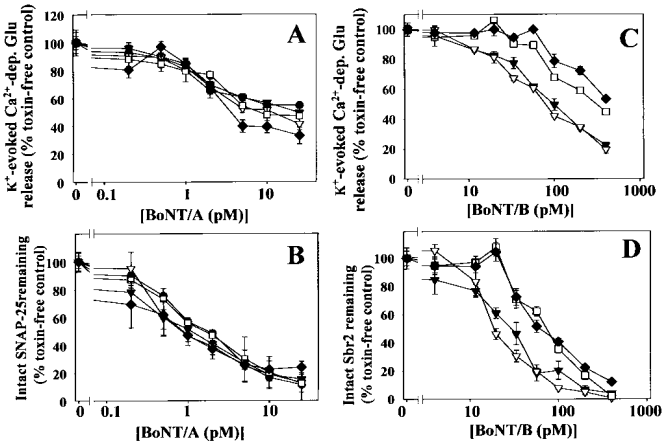

FIG. 3. **Blockade of transmitter release by BoNT/A remains undiminished for more than a month and correlates with persistence of SNAP-25$_A$; the inhibition by BoNT/B partially recovers in this period coincident with incomplete *Sbr*2 replenishment.** Neurons (7–9 DIV) were incubated for 24 h in culture medium with the specified concentrations of BoNT/A (*A* and *B*) or BoNT/B (*C* and *D*). After removal of BoNT/A by washing, neurons were tested immediately (0 days, ●), or the medium was replaced and the cells were cultured for 7 (▼), 14 (▽), 21 (□), or 31 days (◆). In the case of BoNT/B treatment, the neurons were maintained for 2 (▽), 7 (▼), 25 (□), or 28 (◆) days after removing the toxin. *A* and *C*, the extents of blockade of evoked transmitter release were measured (as in Fig. 1 legend) and expressed as the means ± S.D. (n = 3 or 4). *B* and *D*, equal amounts of neuronal protein were immunoblotted with anti-SNAP-25 C-terminal peptide Igs or anti-HV62 Igs (*Sbr*II), and the extents of SNARE cleavage were determined (means ± S.D.; n = 3), as in Fig. 2.

from inhibition of release or replenishment of intact SNAP-25 was detected at any of the BoNT/A concentrations employed (Fig. 3, *A* and *B*), upon weekly monitoring up to 31 days. Additional experiments also demonstrated a lack of significant recovery from blockade of exocytosis 1 month after toxin exposure (data not shown and detailed later). Therefore, the $t_{½\,INH}$ of BoNT/A exceeds 31 days (Table I).

In contrast, BoNT/B induced a concentration-dependent inhibition of transmitter release achieving ~80% blockade (Fig. 3*C*; 100 pM gave 50% inhibition; Table I), together with nearly complete cleavage of *Sbr*2 (Fig. 3*D*), precluding *Sbr*I that is resistant (38). Notably, the truncated *Sbr*2 fragments produced by BoNT/B or BoNT/F, potential contributors to the poisoning, were not visible on Western blots (see below). Upon removing BoNT/B by washing, recovery from inhibition occurred in a time-dependent manner and was accompanied by equivalent partial replacement of intact *Sbr*2 (Fig. 3, *C* and *D*). Exponential decay analysis of data from two BoNT/B dose dependence recovery studies yielded a mean $t_{½\,INH}$ of 9.84 ± 2.12 days (Table I). Therefore, compared with the long lasting BoNT/A and short acting BoNT/E and/F, type B exhibits an intermediate duration of inhibition.

BoNT/C1 Exerts a Long Lasting Inhibition of Exocytosis and Is Neurotoxic—Two days after a 24-h exposure to BoNT/C1, concentration-dependent cleavage of STx1 and SNAP-25 was observed (Fig. 4*B*). A concomitant inhibition of K^+-evoked exocytosis occurred (Fig. 4*A*; 13 pM gave 50% inhibition; Table I) that correlates well with the proteolysis of SNAP-25 but not STx1 (Fig. 4*B*). Whereas there was extensive formation of a SNAP-25 product with a size corresponding to the known N-terminal fragment (residues 1–198) (Fig. 4*D*; see the Introduction), only a residual content of the toxin-truncated N-terminal STx1 product (residues 1–253) (reviewed in Ref. 3) could be detected (Fig. 4*D*). Short exposure to a higher concentration of BoNT/C1 (2 h) resulted in ~80% cleavage of STx1 and SNAP-25; however, the STx1$_{1-253}$ product was no more abundant and only clearly visible if immunoblots were overdeveloped (Fig. 4*D*, *asterisk*); thus, this fragment is rapidly degraded. The possibility that this potential competitor of the SNARE complex contributes to the inhibition of exocytosis is therefore unlikely.

Closer examination of neuron abundance in control and BoNT/C1-treated cultures revealed a dose-dependent effect on survival with 0.33 nM yielding ~50% lethality within 2 days (Fig. 4*C* and Table I). Indeed, C1 lethality was much more apparent after 18 days exposure as indicated by the diminution of BoNT/C1-resistant markers, *Sbr*2 and synaptotagmin1 (data not shown); also, direct neuron counting (Fig. 4*C*) revealed that ~60% of the neurons treated with 33 pM had died. It is apparent that neurons exposed to 10 pM BoNT/C1, showing 47.3 ± 5.3 and 7.7 ± 9.6% cleavage of SNAP-25 and STx1 at 2 days, survived well over the additional 16 days (Fig. 4, *B* and *C*), but those that experienced more extensive initial proteolysis of SNAREs faired poorly. For instance, only ~10% of neurons survived 18 days if their initial intact STx1 content had been diminished by 48.0 ± 8.0% (Fig. 4, *B* and *C*). Despite the difficulties experienced with neuron survival, it was still possible to demonstrate that neither significant recovery from the dose-dependent inhibition of exocytosis (Fig. 4*A*) nor increased contents of intact SNAP-25 and STx1 occurred (Fig. 4*B*). Additional separate experiments lasting either 18 or 25 days post-intoxication (Table I) also demonstrated a lack of significant recovery from BoNT/C1-induced blockade of neuroexocytosis.

[^{35}S]Methionine Pulse Labeling Demonstrates That BoNT/A Protease Has a Long Lifetime in Central Neurons: SNAP-25$_A$ Is Turned Over as Rapidly as the Intact Polypeptide—Failure to recover exocytosis from BoNT/A-intoxicated neurons and persistence of SNAP-25$_A$ suggested that the prolonged inhibition arose from an extended lifetime of SNAP-25$_A$ (known to block exocytosis) (22, 23) and/or the continued activity of the toxin. To address the former possibility, the $t_{½}$ of SNAP-25$_A$ in BoNT/A-pretreated neurons was assessed relative to the intact protein (Fig. 5). The cells were treated for 24 h with BoNT/A and subjected to a 4-h pulse labeling before being harvested (*i.e.* 0 h chase) or chased for the specified times in label-free medium (Fig. 5). After immunoprecipitation of SNAP-25, fluorography revealed time-dependent decreases in [^{35}S]Met-SNAP-25 and -SNAP-25$_A$ (Fig. 5, *A* and *B*). Additionally, immunoblotting of the precipitates with an anti-SNAP-25 antiserum indicated that equivalent amounts of SNAP-25 were analyzed (Fig. 5, *C* and *D*) and that the toxin had proteolyzed a substantial fraction in advance of pulse labeling (Fig. 5*D*). Less than 50% of the newly synthesized [^{35}S]Met-SNAP-25 was proteolyzed by BoNT/A during the 4-h pulse labeling period (Fig. 5*B*; *i.e.* 0 h chase); this contrasts with the >85% cleavage of total SNAP-25

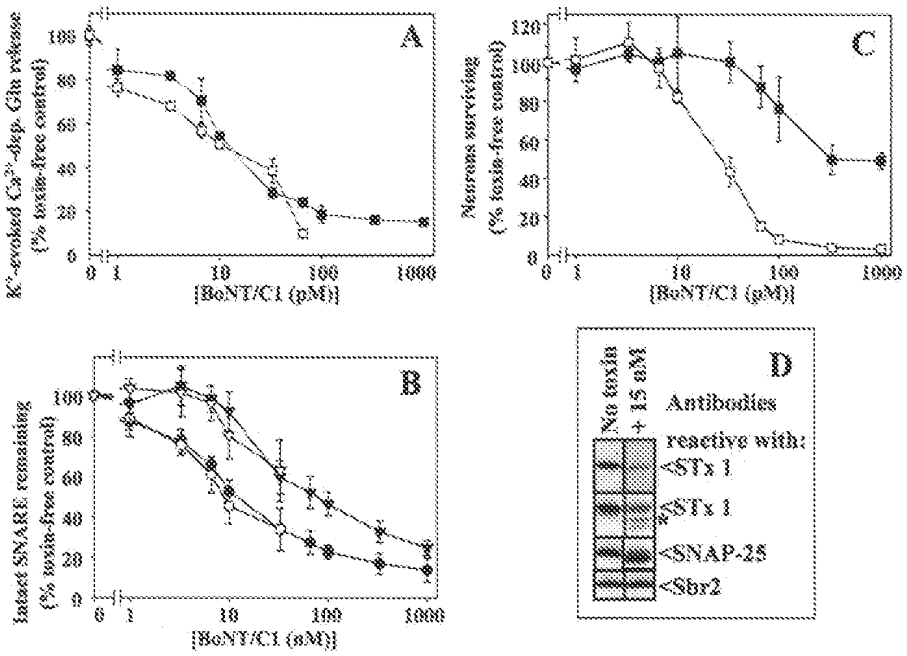

FIG. 4. **BoNT/C1 potently blocks transmitter release for many weeks because of a corresponding persistence of SNAP-25$_{C1}$ and a reduced STx1 content; this serotype can cause cell death.** Neurons (7–9 DIV) were incubated for 20 h in culture medium with the specified concentrations of BoNT/C1. After washing away the toxin, the medium was replaced, and the neurons were assayed 2 (filled symbols) or 18 (open symbols) days later. *A*, the extents of blockade of evoked transmitter release were measured (as in Fig. 1 legend) and expressed as the means ± S.D. (n = 3 or 4). *B*, neuronal protein was immunoblotted using Igs specified in the Fig. 2 legend. The data (means ± S.D.; n = 3) from densitometric scanning of blots were used to determine the extents of cleavage of SNAP-25 (2 days, ●; 18 days, ○) or STx1 (2 days, ▼; 18 days, ▽). *C*, neuron survival at 2 (■) and 18 (□) days was assessed microscopically by counting viable cells. In *D*, following a 2-h exposure in the absence or presence of BoNT/C1, equal amounts of protein were immunoblotted using the specified antibodies (an *asterisk* indicates the toxin-truncated product, STx1 1–253; this was only visible after a prolonged development time).

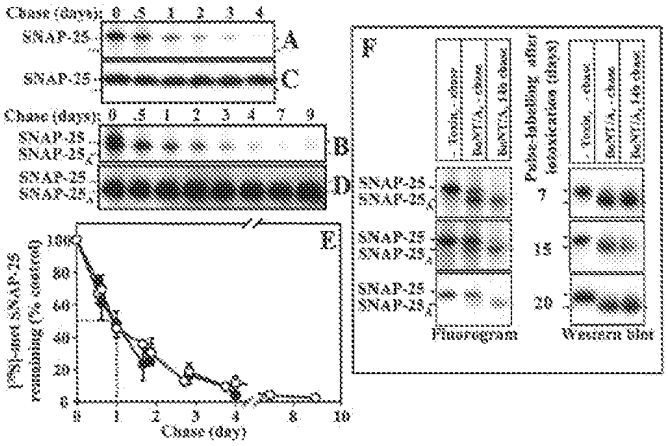

FIG. 5. **[^{35}S]Methionine pulse labeling and immunoprecipitation determine the $t_{½}$ values of intact and SNAP-25$_A$ in developing neurons and demonstrate that BoNT/A protease persists for at least 3 weeks.** Neurons cultured for 7 DIV were exposed for 16 h in the absence (*A* and *C*) or presence (*B* and *D*) of 100 pM BoNT/A prior to [^{35}S]methionine pulse labeling (see "Experimental Procedures"). After the specified chase, the neurons were washed, detergent solubilized, and immunoprecipitated. [^{35}S]Met-labeled SNAP-25 was subjected to SDS-PAGE and fluorography (*A* and *B*) and Western blotting (*C* and *D*) using an anti-SNAP-25 Ig of an alternate species to that used for immunoprecipitation to ensure equal contents in each sample. *E*, the SNAP-25 bands were excised, and their radioactive contents were measured by scintillation counting; the data (± S.D.) are plotted from three experiments each performed in duplicate or triplicate; ●, SNAP-25; ○, SNAP-25A. *F*, neurons cultured for 7 DIV were exposed for 24 h in the absence or presence of 10 pM BoNT/A and then maintained in culture without toxin for the specified period, prior to pulse labeling (with or without chase) and immunoprecipitation of SNAP-25. Immunoadsorbed SNAP-25 was fractionated by SDS-PAGE, and the newly synthesized radiolabeled protein was analyzed by fluorography and Western blotting (see "Experimental Procedures").

detected immunologically (Fig. 5*D*); therefore, newly synthesized SNAP-25 can only represent a minor portion of total SNAP-25. Accurate measurement of radioactivity remaining in the SNAP-25 bands from multiple experiments by scintillation counting (Fig. 5*E*) revealed time-dependent decreases in SNAP-25$_A$ in BoNT/A-treated cells with decay kinetics comparable with the intact protein in toxin-free cells. Extending the chase period beyond 4–8 days revealed a diminution of almost all of the residual [^{35}S]Met-SNAP-25$_A$ detected (Fig. 5, *B* and *E*); the $t_{½}$ values of SNAP-25 and SNAP-25$_A$ extrapolated were 0.89 ± 0.28 and 0.95 ± 0.20 days, respectively (Fig. 5*E*). Therefore, the $t_{½}$ of SNAP-25$_A$ does not account for the longevity of BoNT/A-induced inhibition (N.B. $t_{½\,INH} > 31$ days), at least, in these cultured central neurons.

Next, the persistence of the BoNT/A protease was assessed by examining whether the newly synthesized SNAP-25 was still being proteolyzed at various periods after toxin exposure, visualized using pulse labeling and immunoprecipitation (Fig. 5*F*). Thus, neurons incubated for 24 h in the absence or presence of 10 pM BoNT/A (a concentration sufficient to yield a nearly maximal SNAP-25 cleavage) were cultured in the absence of toxin for the specified period prior to pulse labeling and isolation of SNAP-25, as outlined above (Fig. 5*F*). Additionally, toxin-treated neurons were chased for 14 h to allow sufficient time for the toxin to proteolyze new [^{35}S]Met-SNAP-25. Immunoblotting revealed ~90% cleavage of SNAP-25 in precipitates from type A-treated neurons at all periods examined (Fig. 5*F*). Importantly, 7, 15, and 20 days after intoxication, newly synthesized [^{35}S]Met-SNAP-25 was still efficiently proteolyzed, particularly following the additional 14-h chase (Fig. 5*F*). Reduced neuron survival precluded assessments longer than 3 weeks. Therefore, the notable longevity of BoNT/A-induced inhibition in these cultured central neurons results from persistence of its protease.

Co-exposure of BoNT/A-treated Neurons to Type E Failed to Shorten the Inhibition of Exocytosis: Removal of up to 26 Residues from SNAP-25 Did Not Alter Its Turnover—In view of the observed ability of BoNT/E to foreshorten the paralysis time induced by type A at human and murine neuromuscular junctions (16),[2] neurons were pre-exposed for 24h in the absence or presence of 10 pM type A (Fig. 6*A*, *hatched bars*) or the latter plus 2 nM BoNT/E (Fig. 6*A*, *cross-hatched bars*) prior to assessment of blockade of transmitter release and SNAP-25 cleavage (Fig. 6*B*). The BoNT/A and/E concentrations employed yielded nearly maximal inhibition (Fig. 6*A*) and cleavage of intact SNAP-25 (Fig. 6*B*); SNAP-25$_E$ predominated in doubly treated cells, consistent with the ability of type E to proteolyze SNAP-25$_A$ as efficiently as intact substrate (39). Following a 7-day recovery period, sufficient for nearly complete recovery from the 2 nM BoNT/E used (Fig. 2*A*), evoked release from the

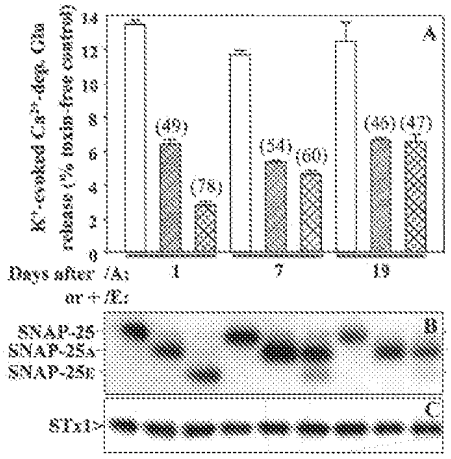

FIG. 6. **Co-exposure of neurons to BoNT/A and BoNT/E does not accelerate recovery of exocytosis or deplete SNAP-25$_A$.** *A*, neurons cultured for 8 DIV were incubated for 1 day in medium in the absence (*open bars*) and presence of 10 pM BoNT/A (*hatched bars*) or 10 pM type A and 2 nM BoNT/E (*cross-hatched bars*). After removal of toxin(s) by washing, the blockade of evoked release was measured at the specified times (means ± S.D.; $n = 4$; see Fig. 1); the values in *brackets* represent percentages of inhibition of transmitter release relative to non-toxin-treated controls. The samples were blotted using anti-SNAP-25 (*B*; clone SMI-81) or anti-STx1 (*C*; clone HPC-1). STx1 immunoblotting confirmed that equivalent amounts of protein were used. The results are representative of two experiments.

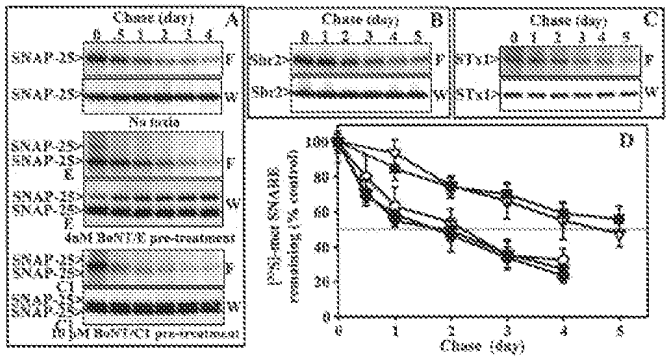

FIG. 7. **Determinations of the $t_{½}$ of intact, and BoNT/E- or BoNT/C1-truncated SNAP-25, *Sbr*2, and STx1 in fully differentiated neurons.** Cerebellar granule cells cultured for 16 DIV were exposed for 16 h in the absence or presence of the specified BoNT prior to [^{35}S]methionine pulse labeling (see "Experimental Procedures"). After the specified chase, the neurons were washed and detergent-solubilized, and their ^{35}S-labeled SNAREs were isolated by immunoprecipitation, using either SMI-81 Ig (*A*; SNAP-25), anti-HV62 Igs (*B*; *Sbr*2), or HPC-1 Ig (*C*; STx-1). The samples were subjected to SDS-PAGE and fluorography (*F*) or Western blotting (*W*) to ensure equal SNARE contents in each sample, using anti-SNARE antibodies generated in species different to those used for immunoprecipitation. Following fluorography, the SNARE bands were excised, and their radioactive contents were measured by scintillation counting; the values for SNAP-25 (●), SNAP-25$_E$ (○), SNAP-25$_{C1}$ (▼), *Sbr*2 (▽), or STx1 (■) are expressed (*D*) relative to their appropriate chase-free controls (means ± S.D.; $n = 3$ or 4).

BoNT/A- and/E- treated neurons remained blocked to a similar extent as in cells exposed to BoNT/A only (~60% *versus* ~54% inhibition). Indeed, even 19 days after co-poisoning, the neurons retained the same level of blockade of release equivalent to that by type A alone (Fig. 6*A*; *i.e.* ~46 and ~47%, respectively). Consistent with the continued blockade of exocytosis by BoNT/A, SNAP-25 in the co-treated neurons existed predominantly in the A-truncated form (Fig. 6*B*); additionally, sequential application of BoNT/E up to 1 month after BoNT/A failed to alleviate blockade of exocytosis by the latter (data not shown).

Pulse-chase studies were performed to compare the turnover rate of intact and BoNT/E- or BoNT/C1-proteolyzed SNAP-25 in fully differentiated neurons (16 DIV) possessing maximal SNARE contents (Fig. 1*C* and data not shown), compared with immature neurons (Fig. 5). Fluorograms demonstrated that a 24-h pretreatment with either 4 nM BoNT/E or 10 pM BoNT/C1 yielded ~85 or ~50% proteolyses of SNAP-25 (Fig. 7*A*; because of the neurotoxicity of BoNT/C1 only submaximal cleavage was possible); immunoblots confirmed that equivalent amounts of SNAP-25 were present in each. Fluorography revealed that ~90% of the newly−synthesized SNAP-25 was rapidly proteolyzed by BoNT/E during the 4-h pulse (Fig. 7*A*; *i.e.* 0-h chase). Conversely, a 1-day chase was necessary for the low dose of BoNT/C1 to cleave the *de novo* synthesized SNAP-25. When the radioactive SNAP-25 remaining after the chase periods were expressed relative to 0 day chases for intact SNAP-25, SNAP-25$_E$ or SNAP-25$_{C1}$, all exhibited similar decay rates ($t_{½}$ of ~2 days) that were notably longer than that of SNAP-25 in younger neurons (Fig. 5).

The Rates of Replacement of Truncated Sbr2 and STx1 in Cerebellar Neurons Are Not Primarily Responsible for the Intermediate or Long Inhibition Exhibited by BoNT/B or BoNT/C1—Because prolonged inhibition by BoNT/B or/C1 may have arisen from slow rates of replacement of cleaved *Sbr*2 or STx1, this possibility was examined. Because mature neurons exhibit maximal SNARE contents from ~13 DIV onwards (only diminished by gradual loss of cell numbers; Fig. 1*C* and data not shown), the rates of SNARE synthesis and degradation must be equivalent in mature neurons (see "Discussion"). Therefore, measurement of the $t_{½}$ of *Sbr*2 and STx1 would also indirectly indicate the rate of SNARE synthesis. Multiple assessments of equivalent immunoprecipitated SNARE samples from different chase periods revealed time-dependent decreases in radiolabeled *Sbr*2 and STx1, using fluorography (Fig. 7, *B* and *C*) and scintillation counting (Fig. 7*D*). A $t_{½}$ of 4–5 days was recorded for *Sbr*2; because the longest chases employed (5 days) failed to yield a 50% reduction of radiolabeled STx1, the $t_{½}$ can only be estimated as ~6 days.

The N-terminal Products from BoNT/B and BoNT/F Cleavage of Sbr2 Are Short-lived: the Stability of BoNT/B Protease Underlies Its Intermediate Duration Blockade of Transmitter Release—Residues 1–76 and 1–58 from Sbr, produced by cleavage with BoNT/B or BoNT/F, can bind tightly to STx1/SNAP-25 heterodimers *in vitro* (12, 13). It is therefore possible that they could prevent binding of intact *Sbr*2, result in competitive inhibition of ternary SNARE complex, and thus block transmitter release. For that reason, it was relevant to examine whether these products can evade cellular degradation and persist. Previous immunoblot analyses of Sbr in toxin-treated synaptosomes, cultured neurons, and neuroendocrine cells failed to detect the cleaved products (Figs. 2 and 3) (26, 38, 40), presumably because of rapid disposal or lack of detection. To evaluate these possibilities, intact recombinant *Sbr*2 was purified and incubated with BoNT/B *in vitro* before being subjected to SDS-PAGE and visualized by protein staining or Western blotting (Fig. 8*A*). As expected, BoNT/B produced two fragments (Fig. 8*A*). Signals were obtained for intact and *Sbr*2$_{1–76}$ (Fig. 8*A*) using a polyclonal anti-Sbr antibody most reactive toward residues 33–45 (40). *Sbr*2$_{77–116}$ was not retained on the nitrocellulose (Fig. 8*A*) and therefore could not be studied. Treatment of neurons with BoNT/B or BoNT/F was found to proteolyze *Sbr*2 as reflected by decreased Sbr immunoreactivity, but no lower M_r bands were detected, suggesting that these fragments are short-lived; thus, their contribution to the exocytotic blockade is precluded.

Having excluded the persistence of potentially inhibitory *Sbr*2 products, as well as slow *Sbr*2 replacement, being the reasons for the prolonged duration of BoNT/B, the longevity of

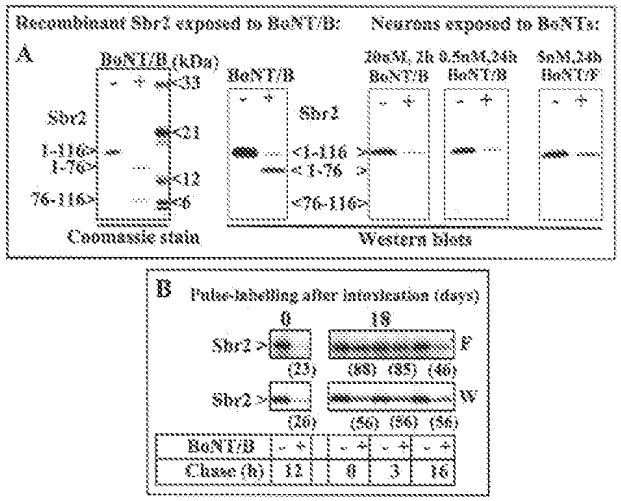

FIG. 8. **The N-terminal products of *Sbr*2 proteolysis by BoNT/B or BoNT/F are short-lived in neurons whereas the BoNT/B protease persists for many weeks.** *A*, recombinant purified *Sbr*2 was incubated with or without BoNT/B using conditions detailed elsewhere (40), fractionated by SDS-PAGE, and visualized by protein staining (2 μg used) or transferred to a nitrocellulose membrane (0.1 μg) for Western blotting, using anti-Sbr Igs (anti-HV62; see "Experimental Procedures"). Primary Ig binding was visualized as in Fig. 1. Additionally, the neurons were treated in the absence or presence of BoNT/B or/F (specified), and equal amounts of protein (20 μg) were Western blotted as outlined above. *B*, neurons cultured for 7 DIV were exposed for 24 h in the absence or presence of 100 pM BoNT/B and then maintained in culture without toxin for the specified period prior to pulse labeling (with or without the specified chase) and immunoprecipitation of *Sbr*2 (as in Fig. 7). Immunoabsorbed *Sbr*2 containing the newly synthesized radiolabeled component was analyzed by fluorography (*F*) and Western blotting (*W*). The *bracketed* values indicate the amounts of the intact Sbr-2 immunoreactivity or radioactivity remaining in BoNT/B-treated samples relative to controls.

its protease was directly examined using pulse labeling and immunoprecipitation. Neurons exposed for 24 h in the absence or presence of 100 pM BoNT/B were pulse labeled immediately (0 days) or cultured for 18 days prior to pulse labeling and *Sbr*2 isolation. This treatment caused 76.2 ± 3.9% (mean ± S.D.; n = 4) and 76.2 ± 5.0% (mean ± S.D.; n = 4) proteolysis of *Sbr*2, immunolabeled or radiolabeled; the values for intact Sbr remaining from a typical experiment are shown in Fig. 8*B*. After 18 days, the amounts of *Sbr*2 cleavage on Western blots noted for BoNT/B-treated neurons had diminished to 43.9 ± 5.4% cleavage (mean ± S.D.; n = 9), consistent with its intermediate persistence noted earlier. Importantly, newly synthesized [^{35}S]Met-*Sbr*2 was still being proteolyzed in a time-dependent manner (Fig. 8*B*); minimal cleavage of [^{35}S]Met-*Sbr*2 occurred following 0- or 3-h chases (11.6 ± 3.8% and 14.1 ± 8.7%, respectively), but after a 16-h chase, significant (53.8 ± 11.5%) cleavage of *Sbr*2 was evident (Fig. 8*B*; values are the means ± S.D.; n = 4). Therefore, persistence of the toxin protease activity is the primary determinant of the longevity of BoNT/B-induced inhibition of exocytosis.

DISCUSSION

The detailed pulse-chase study of native and BoNT-cleaved SNAREs reported herein provides the first unambiguous and direct demonstration of a persistence of BoNT/A protease in central neurons, together with convincing evidence that it is the major factor responsible for prolonged inhibition of neuroexocytosis. Unexpectedly, SNAP-25$_A$ exhibited the same turnover rate as the full-sized protein in cerebellar neurons, in contrast with its reported persistence (2, 24)[2] in peripheral motor nerve endings. Apparently, an exceptional situation must exist in motor nerve terminals *in vivo* (discussed in Refs. 2 and 22),[2] allowing SNAP-25$_A$ to squat at the presynaptic membrane because co-treatment of human or murine endplates with BoNT/A and BoNT/E causes a rapid recovery, equivalent to that of BoNT/E alone (16).[2] The latter would seem to exclude an adequate level of toxin protease persisting—but another study did not detect such a rescue although different conditions (*e.g.* higher toxin dose) were used (41)—though perturbation of the otherwise persistant BoNT/A protease activity or localization following treatment with BoNT/E cannot be precluded. Notably, BoNT/A protease persisted unabated for longer than 1 month in cerebellar neurons, thereby precluding BoNT/E-mediated rescue of exocytosis or depletion of SNAP-25$_A$; the apparent lack of replacement of the latter has been observed previously for spinal cord neurons in culture, although SNAP-25 turnover or protease longevity were not directly measured (25). Similarly, a study performed on cultured neuroendocrine cells observed negligible recovery of catecholamine release or replacement of SNAP-25$_A$ over 2 months following BoNT/A treatment, apparently resulting from protease persistence (22). Therefore, SNAP-25$_A$, but not the E-truncated protein, is retained in motor nerve terminals *in vivo* at the synaptic vesicle release sites; this intriguing dissimilarity with peripheral and central neurons *in vitro* warrants further investigation.

Despite the obvious differences that exist between central cerebellar neurons and motor nerves, many similar neuronal characteristics are conserved; these include common exocytotic mechanisms and proteins, neurite extension, and synapse development. Also, our data reveal that picomolar concentrations of several BoNT serotypes block exocytosis when directly applied to central neurons in culture with potencies matching that observed for motor nerve terminals. *In vivo*, this has not been observed because toxin access to central and nonmotor spinal neurons is largely prevented by anatomical barriers (*e.g.* the blood brain barrier). Moreover, BoNTs do not exhibit detectable levels of retrograde transport, characteristic of TeTx. Preliminary unpublished studies comparing BoNT potency in cultured central neurons and motoneurons have indicated that BoNTs poison cholinergic nerves more rapidly. However, if toxin exposures are performed overnight (*i.e.* when the rate of toxin internalization is not the limiting factor), comparable potencies were observed in both cell types. Most importantly, however, for the purpose of this study concerned with the bases for the different longevities of BoNT serotypes, their relative lifetimes in these neurons are remarkably similar to the distinct durations of neuromuscular paralysis observed *in vivo* for rodents (see the Introduction).

Generation of an avid antibody specific for the LC protease of BoNT/E has allowed tracking of the minute quantities that remain after exposure to nanomolar concentrations. Immunoblotting of cell extracts, after a 2-h treatment with BoNT/E, for several chase periods up to 3 days later revealed that the majority of BoNT/E LC remained as a covalently linked di-chain, inconsistent with its delivery to the cytosol (where it would have been reduced). Therefore, there are at least two pools of toxin in these neurons: endosomal and cytosolic. Although it was necessary to use concentrations of toxins supermaximal to those needed to inhibit exocytosis, nevertheless, the $t_{½\,INH}$ values shown herein correspond to a $t_{½}$ of ~16 h obtained for cell-associated BoNT/E LC immunoreactivity (data not shown).

The different degradation rates found herein for SNAP-25 in developing and mature cerebellar granule neurons (~1 and 2 days, respectively) accord with data from earlier studies (42), which showed that the accumulation of SNAP-25 during development of neurons results from both increased expression and

reduced rates of degradation, processes that stabilize by 14 DIV. The $t_{½}$ values of *Sbr*2 and STx1 in mature neurons (~4.5 and ~6 days) are reported for the first time. These collective findings allowed consideration of the contribution that toxin-truncated SNARE replacement makes to the different durations of transmitter release inhibition by BoNT serotypes. Indeed, the results suggest that the rate of SNAP-25 synthesis governs the length of BoNT/E-induced inhibition. Interestingly, removal of up to 26 C-terminal residues from SNAP-25 does not alter its degradation rate, implicating other signals for regulation of its turnover. The rates of synthesis and degradation of *Sbr*2 must be more rapid in developing neurons relative to the much longer $t_{½}$ of 4–5 days observed for the fully mature protein (*i.e.* analogous to SNAP-25), because a $t_{½\,INH}$ of ~ 2 days was found for BoNT/F in developing neurons. Because another Sbr-cleaving toxin, BoNT/B, persists for much longer ($t_{½\,INH}$ = ~10 days) than the periods required for SNARE synthesis or degradation of the truncated N-terminal fragment, persistence of its protease must account for the prolonged inhibition of exocytosis.

Recent work (43) highlighted the potential risks associated with the clinical use of large quantities of BoNT/B for achieving paralysis of medium length, because of a much reduced safety margin relative to BoNT/A. Although the $t_{½\,INH}$ values determined herein are dependent upon both the times required for removal of the BoNT protease and replacement of cleaved SNARE with intact, protease persistence primarily dictates the larger $t_{½\,INH}$ values measured in neurons treated with BoNT/A, BoNT/C1, or BoNT/B. Attempts by others to examine the $t_{½}$ of the LC of the closely related Clostridial neurotoxin, TeTx, in cultured spinal neurons, found that a highly radio labeled toxin disappeared long before even an initial onset of recovery from blockade of neurotransmission (44); the authors correctly suggest that degradation of TeTx LC ($t_{½}$ = ~6 days) may underlie the slow recovery from neuroinhibition. Indeed, it has been estimated that only 10–100 intracellular toxin molecules are required to inhibit exocytosis (45), precluding straightforward radiolabeled detection; furthermore, this approach does not distinguish between relevant functional toxin protease in the cytosol and that which may reside in other cellular locations (*i.e.* endosomes). Therefore, the methodology used herein for measuring the kinetics of recovery from inhibition offers obvious advantages.

Detailed BoNT dose dependence studies revealed good correlations between losses of intact SNAREs and inhibition of evoked transmitter release, providing a direct demonstration of their involvement in up to 90% of the Ca^{2+}-dependent evoked glutamate exocytosis measured. Note that microanatomical features of motor neurons *in vivo* are not reproduced by neurons in culture (including motoneurons), and they could play important roles in determining the duration, localization, and molecular basis of paralysis (2). However, an imperfect relationship was observed regarding $SNAP\text{-}25_A$ content and inhibition of evoked release in BoNT/A-treated cells; this component of release (~30% of the total) is apparently mediated by $SNAP\text{-}25_A$ because it was reduced by sequential BoNT/E administration. A similar situation has been found in permeabilized neuroendocrine cells (39, 46) and synaptosomes (47).

A small number of patients are primary nonresponders to BoNT/A therapy; also, multiple administrations may gradually elicit immunity in a tiny minority of responders and limit the efficacy of treatment (reviewed in Ref. 14). Therefore, an alternative serotype with the potency and duration of type A is required. In this context, these studies have demonstrated that BoNT/C1 may possess such therapeutic potential (17), except that it has been reported to impair neurite/axonal growth and cause cell death, an effect not ascribable to contamination (Ref. 20 and this work). From the present investigation, it seems that such BoNT/C1 toxicity may result from its proteolysis of STx1 because the dose dependence study revealed that only minimal cleavage of STx1 coincides with the lethal effects, whereas extensive SNAP-25 cleavage was not lethal; also, the $SNAP\text{-}25_{1-198}$ fragment is known to be nonlethal (22). Additional proteolysis of one or more of the other five syntaxin isoforms reported (9) has not been excluded; only STx4 and STx5 are known to be resistant to BoNT/C1 (reviewed in Ref. 3). An essential nonsynaptic vesicle docking fusion role for STx1 in developing neurons is suggested by its notable abundance in immature cerebellar neurons, which are almost devoid of the other SNAREs and lack the functional Ca^{2+}-dependent exocytotic machinery (Fig. 1*C*). In conclusion, this first detailed examination of the molecular basis for the extended action of BoNT/A relative to shorter acting serotypes in neurons has provided novel information that should aid the extension of therapies as well as the development of countermeasures for botulism.

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